Practitioner's Docket No. U 013763-7

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PATENT TRADEMARK OFFICE

CHAPTER II

TRANSMITTAL LETTER TO THE UNITED STATES ELECTED OFFICE (EO/US)

(ENTRY INTO U.S. NATIONAL PHASE UNDER CHAPTER II)

PCT/IL00/00335	7 JUNE 2000	9 JUNE 1999
INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED

A MOLECULAR MARKER BASED ON THE FRK2 (FRUCTOKINASE 2) GENE

TITLE OF INVENTION

1. Ilan LEVIN, 2. Arthur SCHAFFER; 3. Felix CINCAREVSKY

APPLICANT(S)

Box PCT
Assistant Commissioner for Patents
Washington D.C. 20231
ATTENTION: EO/US

NOTE. The completion of those filing requirements that can be made at a time later than 30 months from the priority date results from the Commissioner exercising his judgment under the authority granted under 35 USC 371(d). The filing receipt will show the actual date of receipt of the last item completing the entry into the national phase See 37 C.F.R.

CERTIFICATION UNDER 37 C.F.R. 1.10*
*(Express Mail label number is **mandatory**.)*
(Express Mail certification is optional.)

I hereby certify that this correspondence and the documents referred to as attached therein are being deposited with the United States Postal Service on this date DECEMBER 7, 2001, in an envelope as "Express Mail Post Office to Addressee," Mailing Label Number EV011019569US, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

CONNIE YANNOTTI
(type or print name of person mailing paper)

Signature of person mailing paper

WARNING: *Certificate of mailing (first class) or facsimile transmission procedures of 37 C F R 1 8 cannot be used to obtain a date of mailing or transmission for this correspondence*

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*"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct 24, 1996, 60 Fed Reg 56,439, at 56,442*

§1.491 which states. "An international application enters the national state when the applicant has filed the documents and fees required by 35 USC 371(c) within the periods set forth in § 1.494 and § 1 495."

WARNING: *Where the items are those which can be submitted to complete the entry of the international application into the national phase are subsequent to 30 months from the priority date the application is still considered to be in the international state and if mailing procedures are utilized to obtain a date the express mail procedure of 37 C.F R §1 10 must be used (since international application papers are not covered by an ordinary certificate of mailing - See 37 C F R §1.8.*

NOTE. Documents and fees must be clearly identified as a submission to enter the national state under 35 USC 371 otherwise the submission will be considered as being made under 35 USC 111. 37 C.F.R § 1 494(f)

1. Applicant herewith submits to the United States Elected Office (EO/US) the following items under 35 U.S.C. 371:

   a. [X] This express request to immediately begin national examination procedures (35 U.S.C. 371(f)).

   b. [X] The U.S. National Fee (35 U.S.C. 371(c)(1)) and other fees (37 C.F.R. § 1.492) as indicated below:

2.Fees

CLAIMS FEE	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
[ ]*	TOTAL CLAIMS	18- 20 =		x $ 18.00 =	$
	INDEPENDENT CLAIMS	5- 3 =		x $ 84.00 =	NOT PAID
	MULTIPLE DEPENDENT CLAIM(S) (if applicable) + $280.00				
BASIC FEE**	[X] U.S. PTO WAS INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where an International preliminary examination fee as set forth in § 1.482 has been paid on the international application to the U.S. PTO: [X] and the international preliminary examination report states that the criteria of novelty, inventive step (non-obviousness) and industrial activity, as defined in PCT Article 33(2) to (4) have been satisfied for all the claims presented in the application entering the national stage (37 CFR 1.492(a)(4)) ..... $100.00 [ ] and the above requirements are not met (37 CFR 1.492(a)(1)) ..... $710.00 [ ] U.S. PTO WAS NOT INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where no international preliminary examination fee as set forth in § 1.482 has been paid to the U.S. PTO, and payment of an international search fee as set forth in § 1.445(a)(2) to the U.S. PTO: [ ] has been paid (37 CFR 1.492(a)(2)) ..... $740.00 [ ] has not been paid (37 CFR 1.492(a)(3)) ..... $1,040.00 [ ] where a search report on the international application has been prepared by the European Patent Office or the Japanese Patent Office (37 CFR 1.492(a)(5)) $890.00				
	Total of above Calculations				=100.00
SMALL ENTITY	Reduction by ½ for filing by small entity, if applicable. Statement may also be filed. (note 37 CFR 1.9, 1.27, 1.28)				-50.00
	Subtotal				50.00
	Total National Fee				$50.00
	Fee for recording the enclosed assignment document $40.00 (37 CFR 1.21(h)). (See Item 13 below). See attached "ASSIGNMENT COVER SHEET".				
TOTAL	Total Fees enclosed				$50.00

*See attached Preliminary Amendment Reducing the Number of Claims.

i. [X] A check in the amount of $50.00 to cover the above fees is enclosed.
ii. [ ] Please charge Account No. _____ in the amount of $ _____.
A duplicate copy of this sheet is enclosed.

**WARNING: *"To avoid abandonment of the application the applicant shall furnish to the United States Patent and Trademark Office not later than the expiration of 30 months from the priority date. * * * (2) the basic national fee (see § 1.492(a)) The 30-month time limit may not be extended." 37 C F.R § 1 495(b)*

WARNING: *If the translation of the international application and/or the oath or declaration have not been submitted by the applicant within thirty (30) months from the priority date, such requirements may be met within a time period set by the Office. 37 C F.R § 1.495(b)(2). The payment of the surcharge set forth in § 1.492(e) is required as a condition for accepting the oath or declaration later than thirty (30) months after the priority date. The payment of the processing fee set forth in § 1 492(f) is required for acceptance of an English translation later than thirty (30) months after the priority date. Failure to comply with these requirements will result in abandonment of the application. The provisions of § 1 136 apply to the period which is set Notice of Jan. 3, 1993, 1147 O G 29 to 40.*

3. [X] A copy of the International application as filed (35 U.S.C. 371(c)(2)):

NOTE. *Section 1.495 (b) was amended to require that the basic national fee and a copy of the international application must be filed with the Office by 30 months from the priority date to avoid abandonment "The International Bureau normally provides the copy of the international application to the Office in accordance with PCT Article 20 At the same time, the International Bureau notifies applicant of the communication to the Office In accordance with PCT Rule 47.1, that notice shall be accepted by all designated offices as conclusive evidence that the communication has duly taken place. Thus, if the applicant desires to enter the national stage, the applicant normally need only check to be sure the notice from the International Bureau has been received and then pay the basic national fee by 30 months from the priority date." Notice of Jan 7, 1993, 1147 O G 29 to 40, at 35-36. See item 14c below*

a. [ ] is transmitted herewith.
b. [ ] is not required, as the application was filed with the United States Receiving Office.
c. [X] has been transmitted
i. [X] by the International Bureau.
Date of mailing of the application (from form PCT/IB/308): _____.
ii. [ ] by applicant on _____.
Date

4. [X] A translation of the International application into the English language (35 U.S.C. 371(c)(2)):
a. [ ] is transmitted herewith.
b. [X] is not required as the application was filed in English.
c. [ ] was previously transmitted by applicant on _____.
Date
d. [ ] will follow.

5. [X] Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. 371(c)(3)):

NOTE: The Notice of January 7, 1993 points out that 37 C F R. § 1.495(a) was amended to clarify the existing and continuing practice that PCT Article 19 amendments must be submitted by 30 months from the priority date and this deadline may not be extended. The Notice further advises that, "The failure to do so will not result in loss of the subject matter of the PCT Article 19 amendments. Applicant may submit that subject matter in a preliminary amendment filed under section 1.121. In many cases, filing an amendment under section 1.121 is preferable since grammatical or idiomatic errors may be corrected " 1147 O.G. 29-40, at 36

a. [ ] are transmitted herewith.
b. [ ] have been transmitted
   i. [ ] by the International Bureau.
   Date of mailing of the amendment (from form PCT/IB/308): _____.
   ii. [ ] by applicant on _____ (Date).
c. [X] have not been transmitted as
   i. [X] applicant chose not to make amendments under PCT Article 19.
   Date of mailing of Search Report (from form PCT/ISA/210): _____.
   ii. [ ] the time limit for the submission of amendments has not yet expired. The amendments or a statement that amendments have not been made will be transmitted before the expiration of the time limit under PCT Rule 46.1.

6. [X] A translation of the amendments to the claims under PCT Article 19 (38 U.S.C. 371(c)(3)):
a. [ ] is transmitted herewith.
b. [ ] is not required as the amendments were made in the English language.
c. [X] has not been transmitted for reasons indicated at point 5(c) above.

7. [ ] A copy of the international examination report (PCT/IPEA/409)
[ ] is transmitted herewith.
[ ] is not required as the application was filed with the United States Receiving Office.

8. [ ] Annex(es) to the international preliminary examination report
a. [ ] is/are transmitted herewith.
b. [ ] is/are not required as the application was filed with the United States Receiving Office.

9. [ ] A translation of the annexes to the international preliminary examination report
a. [ ] is transmitted herewith.
b. [ ] is not required as the annexes are in the English language.

10. [X] An oath or declaration of the inventor (35 U.S.C. 371(c)(4)) complying with 35 U.S.C. 115
    a. [ ] was previously submitted by applicant on _____ (Date).
    b. [ ] is submitted herewith, and such oath or declaration
        i. [ ] is attached to the application.
        ii. [ ] identifies the application and any amendments under PCT Article 19 that were transmitted as stated in points 3(b) or 3(c) and 5(b); and states that they were reviewed by the inventor as required by 37 C.F.R. 1.70.
    c. [X] will follow.

Other document(s) or information included:

11. [X] An International Search Report (PCT/ISA/210) or Declaration under PCT Article 17(2)(a):
    a. [ ] is transmitted herewith.
    b. [ ] has been transmitted by the International Bureau.
    Date of mailing (from form PCT/IB/308): _____.
    c. [ ] is not required, as the application was searched by the United States International Searching Authority.
    d. [X] will be transmitted promptly upon request.
    e. [ ] has been submitted by applicant on _____ (Date).

12. [X] An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98:
    a. [ ] is transmitted herewith.
    Also transmitted herewith is/are:
    [ ] Form PTO-1449 (PTO/SB/08A and 08B).
    [ ] Copies of citations listed.
    b. [X] will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. 371(c).
    c. [ ] was previously submitted by applicant on _____ (Date).

13. [ ] An assignment document is transmitted herewith for recording.

A separate [ ] "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or [ ] FORM PTO 1595 is also attached.

_____

_____

_____

14. [X] Additional documents:
    a. [ ] Copy of request (PCT/RO/101)
    b. [X] International Publication No. WO 00/75277
        i. [X] Specification, claims and drawing
        ii. [ ] Front page only
    c. [ ] Preliminary amendment (37 C.F.R. § 1.121)
    d. [ ] Other

15. [X] The above checked items are being transmitted
    a. [X] before 30 months from any claimed priority date.
    b. [ ] after 30 months.

16. [ ] Certain requirements under 35 U.S.C. 371 were previously submitted by the applicant on _____, namely:

AUTHORIZATION TO CHARGE ADDITIONAL FEES

WARNING: *Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges if extra claims are authorized*

NOTE: "A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C.F.R. § 1 136(a)(3).

NOTE: "Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1.26(a).

[X] The Commissioner is hereby authorized to charge the following additional fees that may be required by this paper and during the entire pendency of this application to Account No. 12-0425.

[X] 37 C.F.R. 1.492(a)(1), (2), (3), and (4) (filing fees)

WARNING: *Because failure to pay the national fee within 30 months without extension (37 C F.R § 1.495(b)(2)) results in abandonment of the application, it would be best to always check the above box.*

[ ] 37 C.F.R. 1.492(b), (c) and (d) (presentation of extra claims)

NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must

only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C F R § 1.492(d)), it might be best not to authorize the PTO to charge additional claim fees, except possible when dealing with amendments after final action

[X] 37 C.F.R. 1.17 (application processing fees)
[X] 37 C.F.R. 1.17(a)(1)-(5)(extension fees pursuant to § 1.136(a).
[X] 37 C.F.R. 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. 1.311(b))

NOTE *Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C F R § 1.311(b)*

NOTE *37 C.F R. 1.28(b) requires "Notification of any change in loss of entitlement to small entity status must be filed in the application . . . prior to paying, or at the time of paying . . . issue fee." From the wording of 37 C F R § 1.28(b): (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.*

[ ] 37 C.F.R. § 1.492(e) and (f) (surcharge fees for filing the declaration and/or filing an English translation of an International Application later than 30 months after the priority date).

SIGNATURE OF PRACTITIONER

Reg. No.: 20,302

Julian H. Cohen
(type or print name of practitioner)

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New York, N.Y. 10023

10 Rec'd PCT/PTO 26 APR 2002

Practitioner's Docket No. U 013763-7 ***PATENT***

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

PCT/IL00/00335	7 JUNE 2000	9 JUNE 1999
INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED

A MOLECULAR MARKER BASED ON THE FRK2 (FRUCTOKINASE 2) GENE
TITLE OF INVENTION

ILAN **LEVIN**, ARTHUR **SCHAFFER**, FELIX **CINCAREVSKY**
APPLICANT(S)

BOX PCT
Assistant Commissioner for Patents
Washington, D.C. 20231

WRITTEN ASSERTION OF SMALL ENTITY STATUS

This is written assertion on the basis of:

☐ personal knowledge;

☐ applicant's letter of _____;

☒ applicant's agent's letter of December 6, 2001; or

☐ other _____

by a practitioner (not necessarily of record) that the above application is entitled to small entity status and, therefore, fees.

CERTIFICATION UNDER 37 C.F.R. 1.8(a) and 1.10*
(When using Express Mail, the Express Mail label number is mandatory; Express Mail certification is optional.)

I hereby certify that, on the date shown below, this correspondence is being:

MAILING

☒ deposited with the United States Postal Service in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

37 C.F.R. 1.8(a)

☐ with sufficient postage as first class mail.

37 C.F.R. 1.10*

☒ as "Express Mail Post Office to Address" Mailing Label No. EV011021899US (mandatory)

TRANSMISSION

☐ transmitted by facsimile to the Patent and Trademark Office.

Date: April 26, 2002

Signature

IBIS CARRILLO
(type or print name of person certifying)

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"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will not be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.

NOTE: *"To establish small entity status after the payment of the basic filing or national stage fee as a non-small entity, a written assertion of small entity status is required to be submitted." Notice of September 8, 2000, 65 Fed Reg. 54604, at 54609.*

NOTE: *37 C.F.R. § 1.27(c)(1): "Assertion by writing. Small entity status may be established by a written assertion of entitlement to small entity status. A written assertion must:*

(i) Be clearly identifiable;

(ii) Be signed (see paragraph (c)(2) of this section); and

(iii) Convey the concept of entitlement to small entity status, such as by stating that applicant is a small entity, or that small entity status is entitled to be asserted for the application or patent. While no specific words or wording are required t assert small entity status, the intent to assert small entity status must be clearly indicated in order to comply with the assertion requirement "

NOTE: *37 C.F.R. § 1.27(c)(2): "Parties who can sign and file the written assertion. The written assertion can be signed by:*

(i) One of the parties identified in § 1.33.(b) (e.g. an attorney or agent registered with the Office). § 3.73(b) of this chapter notwithstanding, who can also file the written assertion;

(ii) At least one of the individuals identified as an inventor (even though a § 1.63 executed oath or declaration has not been submitted), notwithstanding § 1.33(b)(4), who can also file the written assertion pursuant to the exception under § 1.33(b) of this part; or

(iii) An assignee of an undivided part interest, notwithstanding §§ 1.33(b(3) and 3.73(b) of this chapter, but the partial assignee cannot file the assertion without resort to a party identified under § 1.33(b) of this part."

35 C.F.R. § 1.33(b):
(b) Amendment and other papers. Amendments and other papers, except for written assertions pursuant to § 1.27(c)(2)(ii) of this part, filed in the application must be signed by:

(1) A registered attorney or agent of record appointed in compliance with § 1.34(b);

(2) A registered attorney or agent not of record who acts in a representative capacity under the provisions of § 1.34(a);

(3) An assignee as provided for under § 3.71(b) of this chapter; or

(4) All of the applicants (§ 1.41(b)) for patent, unless there is an assignee of the entire interest and such assignee has taken action in the application in accordance with § 3.71 of this chapter.

Respectfully submitted,

Clifford J. Mass
c/o Ladas & Parry
26 West 61st Street
New York, N. Y. 10023

A MOLECULAR MARKER BASED ON THE FRK2 (FRUCTOKINASE 2) GENE

FIELD OF THE INVENTION

The present invention relates generally to a method of breeding tomatoes having superior taste characteristics and to tomatoes having superior taste characteristics, and particularly to a molecular marker for a gene determining the fructose to glucose ratio in mature tomato fruit.

BACKGROUND OF THE INVENTION

Taste characteristics are a major determinant of fruit quality for both processing and fresh market tomatoes (see Stevens, M.A. 1986. Inheritance of tomato fruit quality components. Plant Breeding Reviews 4: 274-310) One of the major components of taste in tomatoes is soluble sugar content. The soluble sugar content of all known commercial cultivars of tomatoes (*Lycopersicon esculentum* Mill.) primarily includes the hexose sugars glucose and fructose in near-equimolar ratios (1:1 to 1:1.3) (see Davies J.N. and Hobson G.E. 1981. The constituents of tomato fruit- the influence of environment, nutrition and genotype, CRC Critical Review Food Science and Nutrition, 15:205-280; Davies J.N. and Kempton, R.J. 1975. Changes in the individual sugars of tomato fruit during ripening. J. Sci. Fd. Agric. 26: 1103-1110). In commercial *Lycopersicon esculentum* cultivars the disaccharide sucrose is also present but at concentrations rarely exceeding 0.5% on a fresh weight basis. Certain wild species of *Lycopersicon*, such as *L. hirsutum*, accumulate high concentrations of sucrose, which may reach 4% on a fresh weight basis (see Miron, D. and Schaffer, A.A. 1991. SPS, SS and invertase activities in developing fruit of *Lycopersicon esculentum* and the sucrose accumulating *L. hirsutum*. Plant Physiol. 95: 623-627). In the presence of high sucrose, these fruit accumulate low levels of the hexoses fructose and glucose, typically less than 1% each on a fresh weight basis (Davies J. N. On the Occurrence of Sucrose in *Lycopersicon* Fruit and its Nature, Nature, Vol. 266, 586-587, 1966). However, in these fruit the ratio of fructose to glucose is unusually high, more than 1.5:1.

Typically, plant breeders seek to improve the sweetness component of tomato flavor by increasing total soluble solids (TSS), measured by refractometric determination of a sample of juice and expressed as Brix. This measurement however does not differentiate between the component sugars. Fructose is significantly sweeter than both glucose and sucrose (see Biester, A.M., 1925. Carbohydrate studies: I. Relative sweetness of pure sugars. Amer. J. Physiology 73: 387-400). giving a tomato with a relatively high fructose content distinct advantages in terms of superior taste characteristics.

Tomatoes with high fructose to glucose ratios have been developed, using a method of selection described in applicant/assignee's US Patent application 5,817,913, the disclosure of which is incorporated herein by reference. In summary, this method consists of hybridizing a tomato plant of the *L. esculentum* species with a plant of the *L. hirsutum* species and in the subsequent progenies selection of mature fruit with fructose/glucose ratios of more than 1.8, together with fructose levels more than 1.3% on a fresh weight basis. The analysis of mature fruit sugars in the described method is via direct chemical analysis of the fruit sugars, for example by chromatographic separation of individual sugars.

Molecular markers have been used as a method of selection in plant and animal breeding, with obvious advantages (see Hillel J., Schaap T., Haberfeld A., Jeffreys A.J., Plotzky Y., Cahaner A. and Lavi U. 1990. Genomic selection: application of DNA fingerprints for efficient gene introgression. Genetics, 124:783-789; Tanksley, S.D., Ganal, M.W., Prince, J.P. et al. 1992. High density molecular linkage maps of the tomato and potato genomes. Genetics, 132: 1141-1160; Williamson V.M., Ho J.-Y., Wu F.F., Miller N. and Kaloshian I.. 1994. A PCR-based marker tightly linked to the nematode resistance gene, *Mi*, in tomato. Theor. Appl. Genet., 87:757-763; Chagu'e V., Mercier J.C., Gu'enard M., de Courcel A., and Vedel F. 1996. Identification and mapping on chromosome 9 of RAPD markers linked to *Sw-5* in tomato by bulked segregant analysis. Theor. Appl. Genet., 92:1045-1051). Several strategies to modulate sugar concentration and profile in ripe tomato fruit have been explored, including genetic approaches. However, precision breeding towards such directions involves assessment of reducing sugars carried out by HPLC (high pressure liquid chromatography) that is expensive and time consuming. DNA markers could potentially alleviate this problem, enabling the identification and selection of genetic material at the seedling stage, thus reducing significantly effort and time. During recent years, international efforts were invested aiming at the genome mapping of several plant species such as the tomato, potato and maize, using DNA markers (see Helentjaris T., Slocum M., Wright S., Schaefer A. and Neinhuis J. 1986. Construction of genetic linkage maps in maize and tomato using restriction fragment length polymorphisms. Theor. Appl. Genet., 72: 761-769; Tanksley et al., 1992). Apart from being an efficient tool for many breeding and genetic analyses (reviewed by Hillel J., Dunnington , E.A, and Siegel P.B. 1992. DNA markers in poultry breeding and genetic analysis. Poult. Sci. Rev., 4:169-186), DNA markers also provide initial sequence information and probes useful for cloning genes of interest. Recently, there were several successful reports of gene isolation and candidate gene identification in higher

plants by positional cloning (Tanksley, S.D., Ganal, M.W. and Martin, G.B. 1995. Chromosome landing: a paradigm for map-based cloning in plants with large genomes. Trends Genet., 11: 63-68; Folkertsma R.T., Spassova M.I., Prins M., Stevens M.R , Hille J. and Goldbach R.W. 1999 Construction of a bacterial artificial chromosome (BAC) library of *Lycopersicon esculentum* cv. Stevens and its application to physically map the *Sw-5* locus. Molecular Breeding 5:197-207)

Molecular linkage maps are largely composed of restriction fragment length polymorphism (RFLP) markers. RFLP analysis require a cloned probe, cleavage of genomic DNA with restriction endonucleases and time consuming DNA transfer, labeling and hybridization steps. More efficient polymorphism assays can be obtained from the polymerase chain reaction (PCR), that requires a substantially smaller amount of the analyzed DNA as compared to RFLP analysis (see Saiki R.K., Scharf S., Faloona F.A., Mullis K.B., Horn G.T., Erlich H.A. and Arnheim N. 1985. Enzymatic amplification of b-globin sequences and restriction site analysis for the diagnosis of sickle cell anemia. Science, 230:1350-1354). Several PCR-based marker identification techniques were developed and found useful in the detection of DNA sequences linked to genes of interest. These techniques include the random amplified polymorphic DNA (RAPD, see Williams J.G.K., Kublik A.R., Livak K.J., Rafalski J.A. and Tingey S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res., 18: 6531-6535), microsatellite or simple sequence repeat analysis (SSR, see Tautz, D. 1989. Hypervariability of simple sequences as a general source for polymorphic DNA markers. Nucl Acids Res., 17: 6463-6471), inter SSR polymorphism using single primers of simple sequence repeats (see Gupata M., Chyi Y.-S., Romero-Severson J. and Owen J.L. 1994. Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple-sequence repeats. Theor. Appl. Genet., 89:998-1006) and the technique of amplified restriction fragment polymorphism analysis (AFLP, see Zabeau M. and Vos P. 1993. Selective restriction fragment amplification: a general method for DNA fingerprinting. European Patent Application 92402629.7 (Publication number: 0 534 858 A1)). The PCR techniques, mentioned above, can detect more subtle sequence polymorphisms than RFLP analysis and require only a small amount of DNA. RAPD and inter SSR analysis are low cost and easy to perform because no prior target DNA sequence information in polymorphic DNA regions is required for its implementation. These techniques, however, share the disadvantage of being able to usually identify only a single allele at any given locus and are therefore unable to discriminate

between homozygous and heterozygous genotypes. AFLP is more expensive to produce, can also usually detect only single allele at any given locus, but has the capacity to detect a much greater number of polymorphic loci in a single assay than other currently available PCR-based techniques Microsatellites or SSR are also expensive to produce because they require allele specific primers, detect only a single polymorphic locus in a single assay but have the advantage of being able to identify more than one allele at any given locus and are therefore able to discriminate between homozygous and heterozygous genotypes.

PCR amplification analysis can be followed by restriction endonuclease cleavage of the amplification products in cases where length polymorphism can not be directly obtained in the PCR analysis of different genotypes. Such PCR analysis followed by endonuclease cleavage is often referred to as Cleaved Amplified Polymorphic Sequences (CAPS, see Konieczny A., and Ausubel F.M. 1993. A procedure for Mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. Plant J. 4:403-410; Jarvis P., Lister C., Szabo V. and Dean C. 1994. Integration of CAPS markers into the RFLP map generated using recombinant inbred lines of *Arabidopsis thaliana*. Plant Mol. Biol 24:685-687), can detect more than one allele at any given locus and is therefore able to discriminate between homozygous and heterozygous genotypes.

In the case of selection for sugar content of mature fruit, a molecular marker has the advantage of allowing for selection at the young seedling stage, in contrast to selection only at the mature fruit stage. Furthermore, selection using a molecular marker eliminates the confounding effects of environmental influences on the plant phenotype which can limit the effectiveness of selection for a phenotypic trait such as mature fruit sugar content.

The enzyme fructokinase (EC 2.7.1.4) is able to phosphorylate fructose using a nucleoside triphosphate, such as ATP, as the substrate donating the phosphate moiety. As such, the enzyme may be able to modulate the ratio of fructose to glucose in plant tissue. At least two genes from *L. esculentum* that encode for divergent fructokinase enzymes, termed Frk1 and Frk2 have been cloned and sequenced (see Kanayama, Y., Dai, N., Granot, D., Petreikov, M., Schaffer, A and Bennett, A.B. 1997. Divergent fructokinase genes are differentially expressed in tomato. Plant Physiology 113: 1379-1384). The sequences for these two *L. esculentum* genes are described as Gene Bank Accessions U64817 and U64818, for Frk1 and Frk2, respectively.

It has been shown (Israel Application No. 121373, PCT Application No. PCT/IL98/00336, published application WO 99/04621) that wild species of *Lycopersicon* may

serve as sources of genetic variation for carbohydrate metabolism which may be utilized in the production of tomato plants producing fruit with modified carbohydrate metabolism and sugar content in the fruit.

In a previous patent application (Israel Application No. 121373, PCT Application No. PCT/IL98/00336, published application WO 99/04621) molecular markers associated with a locus in the tomato genome leading to an increase in fructose to glucose ratio in the mature tomato fruit were described. This locus was termed *Fgr* and is localized on tomato chromosome #4 (Levin, I., Gilboa, N., Yeselson, E , Shen, S. and Schaffer A.A. 1999. *Fgr*, a major locus that modulates fructose to glucose ratio in mature tomato fruit. Theor. Appl. Genet., in press).

SUMMARY OF THE INVENTION

The present invention seeks to provide a molecular marker for an additional gene which is operative to an increased fructose to glucose ratio in mature tomato fruit, as compared to the ratio generally present in standard tomato cultivars. In the present patent application we describe a molecular marker for an additional locus, located on tomato chromosome #6, in which the allele derived from a wild *Lycopersicon* species (*L. hirsutum*), modulates the fructose to glucose ratio in mature tomato fruit. The marker is for the gene Fructokinase 2 (*Frk2*), or for a gene linked to Frk2, whose wild species-derived allele increases the fructose to glucose ratio in mature tomato fruit and interacts with the previously characterized locus (*Fgr*), which is located on tomato chromosome number 4. The newly described marker, or the gene, can be used in conjunction with markers tagging the *Fgr* locus to produce tomato seeds, plants and/or fruit with the desirable characteristic of increased fructose to glucose ratio and to further increase this ratio.

There is thus provided in accordance with a preferred embodiment of the present invention an additional molecular marker for a gene or a gene determining fructose to glucose ratio in mature tomato fruit.

In accordance with a preferred embodiment of the present invention the marker includes an amplification product generated by primers called F2F and F2R primers, the F2F primer including a nucleotide sequence CGCCCGCTGAGTTGAATCTTGATCTT and the F2R primer including a nucleotide sequence CACAAGGACATGGCGGATTCATCATC. These primers are designed based on the nucleotide sequence of the gene encoding *Lycopersicon esculentum* fructokinase 2 (Genebank accession number U64818).

The marker that can be used to distinguish the *Frk2* gene originating from *Lycopersicon esculentum* as opposed to the *Frk2* gene originating from *Lycopersicon hirsutum* can be used to

increase fructose/glucose ratio because the *hirsutum* derived allele of the *Frk2* gene is associated with an increase in fructose to glucose ratio. It is reasonable that the marker, or a similar one, can distinguish the *Frk2* gene originating from *Lycopersicon esculentum* as opposed to the *Frk2* gene originating from other wild *Lycopersicon* species.

Further in accordance with a preferred embodiment of the present invention the marker includes at least part of or is at least part of a nucleotide sequence of the fructokinase 2 gene from *Lycopersicon hirsutum* as follows:

1 CATGGCAGTT AACGGTGCTT CTTCCTCTGG TTTGATCGTC AGTTTCGGTG AGATGTTGAT

61 CGATTTCGTT CCGACAGTCT CCGGCGTATC CCTTGCCGAG GCTCCCGGAT TTTTGAAAGC

121 TCCCGGCGGT GCACCGGCGA ACGTCGCTAT CGCGGTGACG AGGCTCGGAG GGAGGTCGGC

181 GTTCGTCGGG AAACTCGGCG ACGATGAGTT CGGTCACATG CTCGCCGGGA TTCTGAAAAC

241 GAACGGCGTA CAAGCCGATG GAATCAATTT TGACAAGGGC GCCAGGACGG CTTTGGCGTT

301 CGTGACTCTA CGCGCCGACG GAGAGCGTGA GTTTATGTTT TACAGAAATC CCAGTGCCGA

361 TATGTTGCTC ACGCCCGCTG AGTTGAATCT TGATCTTATT AGATCTGCTA AGGTGTTCCA

421 CTATGGATCA ATTAGTTTGA TCGTGGAGCC ATGTAGAGCA GCACATATGA AGGCAATGGA

481 AGTAGCTAAG GAGGCAGGGG CATTGCTCTC TTATGACCCT AACCTTCGTT TGCCGTTGTG

541 GCCTTCAGCA GAAGAAGCCA AGAAGCAAAT CAAGAGCATA TGGGACTCTG CTGATGTGAT

601 CAAGGTCAGC GATGTGGAGC TCGAATTCCT CACTGGAAGC AACAAGATTG ATGATGAATC

661 CGCCATGTCC TTGTGGCATC CTAACTTGAA GCTACTCTTG GTCACTCTTG GTGAAAAGGG

721 TTGCAATTAC TACACCAAGA AATTCCATGG AACCGTTGGA GGATTCCATG TGAAGACTGT

781 TGACACCACT GGAGCTGGTG ATTCTTTTGT TGGTGCCCTT CTAACCAAGA TTGTTGATGA

841 TCAAACCATT CTCGACGATG AAGCAAGGTT GAAGGAAGTA CTTAGGTTTT CATGTGCATG

901 TGGAGCCATC ACTACAACCA AGAAAGGAGC AATCCCAGCT TTGCCTACTG CATCTGAAGC

961 CCTCACTTTG CTCAAGGGAG GAGCATAGAA ACATCATGTT ATCTTTTTTC TTTTTTCCAT

1021 CTTCATATAT TTCCCCCCCT TTATGAGTTT TTTTTAACTT TGAAGCTAGT AGGAAGCCTT

Further in accordance with a preferred embodiment of the present invention the marker includes at least part of or is at least part of the amino acid sequence of the fructokinase 2 gene from *Lycopersicon hirsutum* as follows:

MAVNGASSSGLIVSFGEMLIDFVPTVSGVSLAEAPGFLKAPGGAPANVAIAVTRLGG

RSAFVGKLGDDEFGHMLAGILKTNGVQADGINFDKGARTALAFVTLRADGEREFMF
YRNPSADMLLTPAELNLDLIRSAKVFHYGSISLIVEPCRAAHMKAMEVAKEAGALLS
YDPNLRLPLWPSAEEAKKQIKSIWDSADVIKVSDVELEFLTGSNKIDDESAMSLWHP
NLKLLLVTLGEKGCNYYTKKFHGTVGGFHVKTVDTTGAGDSFVGALLTKIVDDQTI
LDDEARLKEVLRFSCACGAITTTKKGAIPALPTASEALTLLKGGA

Still further in accordance with a preferred embodiment of the present invention the amplification product generated by F2F and F2R is digested with the endonuclease *Eco*R I to generate a restriction fragment length polymorphism that distinguishes between the allele derived from *Lycopersicon hirsutum* and the allele derived from *Lycopersicon esculentum*.

There is also provided in accordance with a preferred embodiment of the present invention a method for breeding tomato plants that produce tomatoes having superior taste characteristics, including the steps of crossing at least one *Lycopersicon esculentum* plant with a *Lycopersicon* spp. to produce hybrid seeds, collecting the hybrid (F_1) seeds, growing plants from the F_1 seeds, pollinating the F_1 plants, collecting the hybrid seeds produced by the F_1 plants, growing plants from the seeds produced by the F_1 plants, measuring glucose and fructose content of ripe fruit produced from the plants grown from the seeds of the F_1 plants, providing a marker that distinguishes a *Frk2* gene originating from *Lycopersicon esculentum* as opposed to a *Frk2* gene originating from a *Lycopersicon* species, the marker being a marker for increased fructose/glucose ratio in tomato fruit, and using the marker to select a tomato plant with tomato fruit having desired characteristics including a fructose to glucose ratio greater than a ratio of standard *Lycopersicon esculentum*.

There is also provided in accordance with a preferred embodiment of the present invention a method for finding a gene, or a promoter region of a gene, that produces tomatoes having superior taste characteristics, including the steps of providing a marker that distinguishes a *Frk2* gene originating from *Lycopersicon esculentum* as opposed to a *Frk2* gene originating from a wild *Lycopersicon* species, the marker being a marker for increased fructose/glucose ratio in tomato fruit and using the marker to find the gene or the promoter region of said gene.

In accordance with a preferred embodiment of the present invention the method further includes cloning the gene

Additionally in accordance with a preferred embodiment of the present invention the

method includes the step of propagating the plants with tomato fruits having the desired characteristics. Alternatively the plants may be propagated by vegetative propagation or by seed.

A tomato plant, tomato fruit and/or tomato seed may be produced in accordance with any of the methods of the present invention.

DETAILED DESCRIPTION OF A PREFERRED EMBODIMENT

Reference is now made to a method for selecting, in a breeding program, tomato plants with the genetic composition that determines that the mature fruit will have a fructose to glucose ratio higher than that found in standard tomato cultivars, on the average. The method of developing the plant material is as described in applicant/assignee's US Patent No. 5,817,913. Reference is now made to the following example which illustrates the invention.

Plant material description and analysis of sugar content in mature fruit

Parental lines of *Lycopersicon esculentum* differing significantly in their fructose to glucose ratio in the mature fruit were selected for this study, together with F_1, F_2 and F_3 populations generated by crossing the two parental lines. The high fructose to glucose ratio breeding line was derived from the introgression of the trait of high fructose to glucose ratio from the wild species *Lycopersicon hirsutum* (LA1777), as described in US Patent No. 5,817,913.

The following procedure was carried out for soluble sugar determination. Fruit portions of about 500 mg fresh weight were placed in 80% ethanol and soluble sugars were extracted from the tissue by heating to 70°C, as described in Miron and Schaffer (1991). Sugars were chromatographically separated by HPLC using a Bio-Rad Fast Carbohydrate column according to manufacturer's directions, as in Miron and Schaffer (1991). Sucrose glucose and fructose were identified by their retention times, refractometrically, and quantified in comparison to sugar standards.

Description of the PCR method, the PCR amplification marker generated by F2F and F2R primers and the analysis of the results

Genomic DNA was extracted from the 2 parental lines with divergent fructose to glucose ratio in the mature fruit and from individual plants of the F_1, F_2 and F_3 populations generated by crossing the two parental lines. The individual plants from the F_2 and F_3 population segregated for the trait of fructose to glucose ratio, the range being 1-2.5 in the F_2 population and 1.1-7.7 in the F_3 population. Individual plants from the F_2 and F_3 populations could therefore be easily

ranked for the trait of fructose to glucose ratio. The genomic DNA was extracted as in Fulton, T.M., Chunwongse, J. and Tanksley, S.D. 1995. Microprep protocol for extraction of DNA from tomato and other herbaceous plants. Plant Molecular Biology Reporter 13: 207-209. In short, 50-100 mg of leaf tissue was ground in the presence of 2.5 parts DNA extraction buffer (0.35 M sorbitol, 0.1 M Tris-base, 5 mM EDTA, pH, 7.5); 2.5 parts nuclei lysis buffer (0.2 M Tris, 0.05 M EDTA, 2 M NaCl, 5% CTAB); 1 part 5% sarkosyl and 0.3 gm sodium bisulfite/100 ml. After incubation at 65 C for 120 min DNA was extracted with chloroform:isoamyl (24:1), precipitated with isopropanol, washed with 70% ethanol, dried and resuspended in ddH_2O.

F2F and F2R primers were synthesized (GibcoBRL, Inc., U.K.). These primers were designed based on the nucleotide sequence of the gene encoding *Lycopersicon esculentum* fructokinase 2 (Genebank accession number U64818). These primers were used in the presence of template DNA to screen by a polymerase amplification reaction for polymorphism between parental lines with divergent fructose to glucose ratios. The PCR products were digested with various restriction endonucleases and *Eco*R I was found to generate such restriction fragment length polymorphism.

Amplification reactions for the *Frk2* locus (25 µl final volume) contained 10 ng template DNA, 25 mM TAPS (pH 9.3 at 25°C), 50 mM KCl, 2mM MgCl2, 1 mM b-mercaptoethanol, 0.2 mM of each of the four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP), 10 ng of each of two primers (F2F and F2R), and 1 unit of thermostable Taq DNA polymerase (SuperNova Taq Polymerase, MADI LTD., Israel). Reactions were carried out in an automated thermocycler (MJ Research Inc., Watertown, Massachusetts, USA). Initial incubation was at 94°C for 1.5 min, followed by 34 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and polymerization at 72°C for 1.5 min. Final polymerization at 72°C was carried out for 7 min after cycles were completed. The amplification products were visualized, after digestion with EcoRI (37^{o}, 1 hour) according to manufacturer's recommendations, (New England Biolabs Inc., Beverly, MA, USA) by electrophoresis in 1.5% agarose gels and detected by staining with ethidium bromide. The genotype of each of the individual plants for the *Fgr* locus was determined as previously described in PCT published application WO 99/04621.

Genotype-phenotype relation

Analyses of variance were carried out using results obtained from the F_2 and F_3

population to determine the effect of association between each of the markers and the trait of fructose to glucose ratio and the percentage of fructose to glucose variation explained by these variation components. The DNA markers obtained were found highly and significantly associated with the trait of fructose to glucose ratio in both F_2 and F_3 populations (Tables 1,2,3 and 4). The association between both markers and the trait of fructose to glucose ratio was highly significant at a high log-of-differences (LOD) score explaining, together with a statistically significant interaction between them 48.5% and 61.9% of the total variation in fructose to glucose ratios observed in the F_2 and the F_3 populations, respectively (Table 2 and 4, respectively).

In conclusion, the results presented indicate that:

1. The DNA marker obtained by the amplification reactions using F2F and F2R primers is highly associated with an additional major gene encoding fructose to glucose ratios in the mature tomato fruits; and

2. The gene identified can directly or indirectly (through an interaction with the *Fgr* locus) modulate fructose to glucose ratios.

Table 1. Association between the fructokinase 2, *Fgr* and the trait of fructose to glucose ratio in F_2 population.

	Fructokinase 2		
Fgr	HH	HE	EE
HH	2.10 ± 0.09^{A}	1.74 ± 0.04^{B}	1.54 ± 0.06^{C}
HE	1.71 ± 0.05^{B}	1.49 ± 0.03^{C}	1.41 ± 0.02^{C}
EE	1.29 ± 0.04^{D}	1.24 ± 0.03^{D}	1.25 ± 0.02^{D}

It is noted that different letters represent statistically significant differences at the 0.05 level of significance The letters E and H represent the derived genotypes of *esculentum* and *hirsutum*, and HE denotes the heterozygote thereof.

Table 2. Analysis of variance estimating the effects of fructokinase 2 locus, *Fgr* locus and the interaction between them on fructose to glucose ratio in the F_2 population.

Source of variation	Sum of squares	df	F ratio	Prob>F
Fructokinase 2 (*Frk2-II*)	1.49	2	20.84	5×10^{-9}
Fgr	6.09	2	84.84	4×10^{-28}
Frk2-II X *Fgr*	0.81	4	5.66	0.000232
Error	8.01	223		R^2=48.5

Table 3. Association between the fructokinase 2, *Fgr* and the trait of fructose to glucose ratio in F_3 population.

	Fructokinase 2		
Fgr	HH	HE	EE
HH	3.81 ± 0.15^{A}	2.26 ± 0.16^{C}	2.01 ± 0.06^{C}
HE	2.88 ± 0.18^{B}	1.76 ± 0.14^{D}	1.71 ± 0.02^{D}
EE	1.68 ± 0.17^{D}	1.30 ± 0.15^{E}	1.37 ± 0.02^{E}

Table 4 Analysis of variance estimating the effects of fructokinase 2 locus, *Fgr* locus and the interaction between them on fructose to glucose ratio in the F_3 population.

Source of variation	Sum of squares	df	F ratio	Prob>F
Fructokinase 2 (*Frk2-II*)	24.11	2	47.94	7×10^{-17}
Fgr	28.88	2	57.44	4×10^{-19}
Frk2-II X *Fgr*	7.88	4	7.83	0.000009
Error	38.22	152		$R^2=61.9$

Sequence of *Lycopersicon hirsutum* fructokinase II gene

Total RNA was extracted from young fruits (2 g fresh weight) of an individual plant homozygous for the fructokinase II allele derived from *Lycopersicon hirsutum* (*Frk2-II* ^{HH}). The RNA extraction was carried out using the TRIzol reagent system (GibcoBRL Life Technologies, Gaithersburg, MD, USA). The total RNA was used as template for first strand cDNA synthesis using the Superscript preamplification system (GibcoBRL Life Technologies, U.K.). The cDNA prepared was used as template in a PCR reaction to amplify four overlapping fragments of the

gene encoding fructokinase II (*Frk2* ^{HH}). The DNA fragments were excised from an agarose gel and purified using the GENECLEAN II kit (BIO 101 Inc., La Jolla CA, USA). The PCR fragments were then cloned into an pGEM-T Easy vector using the pGEM-T and pGEM-T Easy Vector Systems according to the manufacturer recommendations (Promega corporation, Madison, WI, USA). Four independent clones of each of the four amplified fragments were sequenced, based on both the T7 and SP6 complementary primers, using an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

The nucleotide sequence of the fructokinase II gene derived from *Lycopersicon hirsutum* (*Frk2*^{HH}) is as follows:

1 CATGGCAGTT AACGGTGCTT CTTCCTCTGG TTTGATCGTC AGTTTCGGTG AGATGTTGAT

61 CGATTTCGTT CCGACAGTCT CCGGCGTATC CCTTGCCGAG GCTCCCGGAT TTTTGAAAGC

121 TCCCGGCGGT GCACCGGCGA ACGTCGCTAT CGCGGTGACG AGGCTCGGAG GGAGGTCGGC

181 GTTCGTCGGG AAACTCGGCG ACGATGAGTT CGGTCACATG CTCGCCGGGA TTCTGAAAAC

24GAACGGCGTA CAAGCCGATG GAATCAATTT TGACAAGGGC GCCAGGACGG CTTTGGCGTT

301 CGTGACTCTA CGCGCCGACG GAGAGCGTGA GTTTATGTTT TACAGAAATC CCAGTGCCGA

361 TATGTTGCTC ACGCCCGCTG AGTTGAATCT TGATCTTATT AGATCTGCTA AGGTGTTCCA

421 CTATGGATCA ATTAGTTTGA TCGTGGAGCC ATGTAGAGCA GCACATATGA AGGCAATGGA

481 AGTAGCTAAG GAGGCAGGGG CATTGCTCTC TTATGACCCT AACCTTCGTT TGCCGTTGTG

541 GCCTTCAGCA GAAGAAGCCA AGAAGCAAAT CAAGAGCATA TGGGACTCTG CTGATGTGAT

601 CAAGGTCAGC GATGTGGAGC TCGAATTCCT CACTGGAAGC AACAAGATTG ATGATGAATC

661 CGCCATGTCC TTGTGGCATC CTAACTTGAA GCTACTCTTG GTCACTCTTG GTGAAAAGGG

721 TTGCAATTAC TACACCAAGA AATTCCATGG AACCGTTGGA GGATTCCATG TGAAGACTGT

781 TGACACCACT GGAGCTGGTG ATTCTTTTGT TGGTGCCCTT CTAACCAAGA TTGTTGATGA

841 TCAAACCATT CTCGACGATG AAGCAAGGTT GAAGGAAGTA CTTAGGTTTT CATGTGCATG

901 TGGAGCCATC ACTACAACCA AGAAAGGAGC AATCCCAGCT TTGCCTACTG CATCTGAAGC

961 CCTCACTTTG CTCAAGGGAG GAGCATAGAA ACATCATGTT ATCTTTTTTC TTTTTTCCAT

1021 CTTCATATAT TTCCCCCCCT TTATGAGTTT TTTTTAACTT TGAAGCTAGT AGGAAGCCTT

It will be appreciated by persons skilled in the art that the present invention is not limited by what has been particularly shown and described hereinabove. Rather the scope of the present invention includes both combinations and subcombinations of the features described hereinabove as well as modifications and variations thereof which would occur to a person of skill in the art upon reading the foregoing description and which are not in the prior art.

CLAIMS

What is claimed is:

1. A molecular marker that distinguishes a *Frk2* gene originating from *Lycopersicon esculentum* as opposed to a *Frk2* gene originating from a wild *Lycopersicon* species, said marker being a marker for increased fructose/glucose ratio in tomato fruit as compared to a ratio generally present in standard tomato cultivars.

2. A molecular marker for a gene linked to *Frk2* having a wild-species derived allele, whose wild species-derived allele increases fructose to glucose ratio in mature tomato fruit as compared to a ratio generally present in standard tomato cultivars.

3. A molecular marker according to claim 2 wherein said marker is part of the *Frk2* gene.

4. A molecular marker that, upon interaction with another marker that tags a *Fgr* locus located on tomato chromosome number 4, is a marker for increased fructose/glucose ratio in tomato fruit as compared to a ratio generally present in standard tomato cultivars.

5. A molecular marker according to claim 1 that distinguishes a *Frk2* gene originating from *Lycopersicon esculentum* as opposed to a *Frk2* gene originating from *Lycopersicon hirsutum*.

6. A marker according to claim 1 and further comprising an amplification product generated by a primers called F2F and F2R that are further digested with *Eco*R I endonuclease, comprising a nucleotide sequence:

F2F= CGCCCGCTGAGTTGAATCTTGATCTT, and

F2R= CACAAGGACATGGCGGATTCATCATC.

7. A marker according to claim 6 and further comprising a fragment having a nucleotide sequence as follows:

1 CATGGCAGTT AACGGTGCTT CTTCCTCTGG TTTGATCGTC AGTTTCGGTG AGATGTTGAT

61 CGATTTCGTT CCGACAGTCT CCGGCGTATC CCTTGCCGAG GCTCCCGGAT TTTTGAAAGC

121 TCCCGGCGGT GCACCGGCGA ACGTCGCTAT CGCGGTGACG AGGCTCGGAG GGAGGTCGGC

181 GTTCGTCGGG AAACTCGGCG ACGATGAGTT CGGTCACATG CTCGCCGGGA TTCTGAAAAC

241 GAACGGCGTA CAAGCCGATG GAATCAATTT TGACAAGGGC GCCAGGACGG CTTTGGCGTT

301 CGTGACTCTA CGCGCCGACG GAGAGCGTGA GTTTATGTTT TACAGAAATC CCAGTGCCGA

361 TATGTTGCTC ACGCCCGCTG AGTTGAATCT TGATCTTATT AGATCTGCTA AGGTGTTCCA

421 CTATGGATCA ATTAGTTTGA TCGTGGAGCC ATGTAGAGCA GCACATATGA AGGCAATGGA

481 AGTAGCTAAG GAGGCAGGGG CATTGCTCTC TTATGACCCT AACCTTCGTT TGCCGTTGTG

541 GCCTTCAGCA GAAGAAGCCA AGAAGCAAAT CAAGAGCATA TGGGACTCTG CTGATGTGAT

601 CAAGGTCAGC GATGTGGAGC TCGAATTCCT CACTGGAAGC AACAAGATTG ATGATGAATC

661 CGCCATGTCC TTGTGGCATC CTAACTTGAA GCTACTCTTG GTCACTCTTG GTGAAAAGGG

721 TTGCAATTAC TACACCAAGA AATTCCATGG AACCGTTGGA GGATTCCATG TGAAGACTGT

781 TGACACCACT GGAGCTGGTG ATTCTTTTGT TGGTGCCCTT CTAACCAAGA TTGTTGATGA

841 TCAAACCATT CTCGACGATG AAGCAAGGTT GAAGGAAGTA CTTAGGTTTT CATGTGCATG

901 TGGAGCCATC ACTACAACCA AGAAAGGAGC AATCCCAGCT TTGCCTACTG CATCTGAAGC

961 CCTCACTTTG CTCAAGGGAG GAGCATAGAA ACATCATGTT ATCTTTTTTC TTTTTTCCAT

1021 CTTCATATAT TTCCCCCCCT TTATGAGTTT TTTTTAACTT TGAAGCTAGT AGGAAGCCTT

8. A marker according to claim 6 and further comprising a fragment having an amino acid sequence as follows:

MAVNGASSSGLIVSFGEMLIDFVPTVSGVSLAEAPGFLKAPGGAPANVAIAVTRLGG
RSAFVGKLGDDEFGHMLAGILKTNGVQADGINFDKGARTALAFVTLRADGEREFMF
YRNPSADMLLTPAELNLDLIRSAKVFHYGSISLIVEPCRAAHMKAMEVAKEAGALLS
YDPNLRLPLWPSAEEAKKQIKSIWDSADVIKVSDVELEFLTGSNKIDDESAMSLWHP
NLKLLLVTLGEKGCNYYTKKFHGTVGGFHVKTVDTTGAGDSFVGALLTKIVDDQTI
LDDEARLKEVLRFSCACGAITTTKKGAIPALPTASEALTLLKGGA

9. A method for breeding tomato plants that produce tomatoes having superior taste characteristics, comprising the steps of.

crossing at least one *Lycopersicon esculentum* plant with a *Lycopersicon* spp. to produce hybrid seeds;

collecting the hybrid (F_1) seeds;

growing plants from the F_1 seeds;

pollinating the F_1 plants;

collecting the hybrid seeds produced by the F_1 plants;

growing plants from the seeds produced by the F_1 plants;

measuring glucose and fructose content of ripe fruit produced from the plants grown from

the seeds of the F_1 plants;

providing a marker that distinguishes a *Frk2* gene originating from *Lycopersicon esculentum* as opposed to a *Frk2* gene originating from a wild *Lycopersicon* species, said marker being a marker for increased fructose/glucose ratio in tomato fruit; and

using said at least one additional marker to select a tomato plant with tomato fruit having desired characteristics including a fructose to glucose ratio greater than a ratio of standard *Lycopersicon esculentum*.

10 A method for finding a gene that produce tomatoes having superior taste characteristics, comprising the steps of:

providing a marker that distinguishes a *Frk2* gene originating from *Lycopersicon esculentum* as opposed to a *Frk2* gene originating from a wild *Lycopersicon* species, said marker being a marker for increased fructose/glucose ratio in tomato fruit; and

using said at least one additional marker to find said gene.

11. A method for finding a promoter region of a gene that produce tomatoes having superior taste characteristics, comprising the steps of:

providing a marker that distinguishes a *Frk2* gene originating from *Lycopersicon esculentum* as opposed to a *Frk2* gene originating from a wild *Lycopersicon* species, said marker being a marker for increased fructose/glucose ratio in tomato fruit; and

using said at least one additional marker to find a promoter region of said gene.

12. A method according to claim 10 and further comprising cloning said gene.

13. A method according to claims 9 and additionally comprising the step of propagating said plants with tomato fruits having the desired characteristics.

14. A method according to claim 13 wherein the step of propagating includes the step of vegetative propagation.

15. A method according to claim 13 wherein the step of propagating includes the step of propagation by seed.

16 A tomato plant produced according to the method of claim 9.

17 A tomato fruit produced by a tomato plant in accordance with claim 16.

18. A tomato seed which when grown yield a tomato plant in accordance with claim 16.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/75277 A2

(54) Title: A MOLECULAR MARKER BASED ON THE Frk2 (FRUCTOKINASE 2) GENE

(57) Abstract: A molecular marker that distinguishes a *Frk2* gene originating from *Lycopersicon esculentum* as opposed to a *Frk2* gene originating from a wild *Lycopersicon* species, said marker being a marker for increased fructose/glucose ratio in tomato fruit as compared to a ratio generally present in standard tomato cultivars.

Practitioner's Docket No. U 013763-7 ***PATENT***

Optional Customer No. Bar Code

00140

PATENT TRADEMARK OFFICE

COMBINED DECLARATION AND POWER OF ATTORNEY

(ORIGINAL, DESIGN, NATIONAL STAGE OF PCT, SUPPLEMENTAL, DIVISIONAL, CONTINUATION, OR C-I-P)

As a below named inventor, I hereby declare that:

TYPE OF DECLARATION

This declaration is of the following type:

(check one applicable item below)

[ ] original.
[ ] design.

NOTE. With the exception of a supplemental oath or declaration submitted in a reissue, a supplemental oath or declaration is not treated as an amendment under 37 CFR 1.312 (Amendments after allowance). M.P.E.P. Section 714.16, 7th Ed.

[ ] supplemental.

NOTE. If the declaration is for an International Application being filed as a divisional, continuation or continuation-in-part application, do not check next item; check appropriate one of last three items.

[x] national stage of PCT.

NOTE: If one of the following 3 items apply, then complete and also attach ADDED PAGES FOR DIVISIONAL, CONTINUATION OR C-I-P.

NOTE: See 37 C.F.R. Section 1.63(d) (continued prosecution application) for use of a prior nonprovisional application declaration in the continuation or divisional application being filed on behalf of the same or fewer of the inventors named in the prior application.

[ ] divisional.
[ ] continuation.

NOTE: Where an application discloses and claims subject matter not disclosed in the prior application, or a continuation or divisional application names an inventor not named in the prior application, a continuation-in-part application must be filed under 37 C.F.R. Section 1.53(b) (application filing requirements-nonprovisional application).

[ ] continuation-in-part (C-I-P).

INVENTORSHIP IDENTIFICATION

WARNING: *If the inventors are each not the inventors of all the claims, an explanation of the facts, including the ownership of all the claims at the time the last claimed invention was made, should be submitted.*

My residence, post office address and citizenship are as stated below, next to my name. I believe that I am the original, first and sole inventor *(if only one name is listed below)* or an original, first and joint inventor *(if plural names are listed below)* of the subject matter that is claimed, and for which a patent is sought on the invention entitled:

TITLE OF INVENTION

A MOLECULAR MARKER BASED ON THE FRK2 (FRUCTOKINASE 2) GENE

SPECIFICATION IDENTIFICATION

The specification of which:

(complete (a), (b), or (c))

(a) [ ] is attached hereto.

NOTE: *"The following combinations of information supplied in an oath or declaration filed on the application filing date with a specification are acceptable as minimums for identifying a specification and compliance with any one of the items below will be accepted as complying with the identification requirement of 37 C.F.R. Section 1.63:*

"(1) name of inventor(s), and reference to an attached specification which is both attached to the oath or declaration at the time of execution and submitted with the oath or declaration on filing;

"(2) name of inventor(s), and attorney docket number which was on the specification as filed; or

"(3) name of inventor(s), and title which was on the specification as filed."

Notice of July 13, 1995 (1177 O.G. 60).

(b) [ ] was filed on _____, [ ] as Application No. _____
[ ] and was amended on _____ (if applicable).

NOTE: *Amendments filed after the original papers are deposited with the PTO that contain new matter are not accorded a filing date by being referred to in the declaration. Accordingly, the amendments involved are those filed with the application papers or, in the case of a supplemental declaration, are those amendments claiming matter not encompassed in the original statement of invention or claims. See 37 C.F.R. Section 1.67.*

NOTE: *"The following combinations of information supplied in an oath or declaration filed after the filing date are acceptable as minimums for identifying a specification and compliance with any one of the items below will be accepted as complying with the identification requirement of 37 C.F.R. Section 1.63:*

(A) application number (consisting of the series code and the serial number, e.g., 08/123,456);
(B) serial number and filing date;
(C) attorney docket number which was on the specification as filed;
(D) title which was on the specification as filed and reference to an attached specification which is both attached to the oath or declaration at the time of execution and submitted with the oath or declaration; or
(E) title which was on the specification as filed and accompanied by a cover letter accurately identifying the application for which it was intended by either the application number (consisting of the series code and the serial number, e.g., 08/123,456), or serial number and filing date. Absent any statement(s) to the contrary, it will be presumed that the application filed in the PTO is the application which the inventor(s) executed by signing the oath or declaration.

M.P.E.P. Section 601.01(a), 7th ed.

(c) [x] was described and claimed in PCT International Application No. PCT/IL00/00335 filed on 7 JUNE 2000 and as amended under PCT Article 19 on _____ *(if any)*.

SUPPLEMENTAL DECLARATION (37 C.F.R. Section 1.67(b))

(complete the following where a supplemental declaration is being submitted)

[ ] I hereby declare that the subject matter of the

[ ] attached amendment
[ ] amendment filed on _____.

was part of my/our invention and was invented before the filing date of the original application, above identified, for such invention.

ACKNOWLEDGMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information, which is material to patentability as defined in 37, Code of Federal Regulations, Section 1.56,

(also check the following items, if desired)

[ ] and which is material to the examination of this application, namely, information where there is a substantial likelihood that a reasonable Examiner would consider it important in deciding whether to allow the application to issue as a patent, and

[ ] in compliance with this duty, there is attached an information disclosure statement, in accordance with 37 C.F.R. Section 1.98.

PRIORITY CLAIM (35 U.S.C. Section 119(a)-(d))

NOTE: "The claim to priority need be in no special form and may be made by the attorney or agent if the foreign application is referred to in the oath or declaration as required by Section 1.63. The claim for priority and the certified copy of the foreign application specified in 35 U.S.C. Section 119(b) must be filed in the case of an interference (Section 1.630), when necessary to overcome the date of a reference relied upon by the examiner, when specifically required by the examiner, and in all other situations, before the patent is granted. If the claim for priority or the certified copy of the foreign application is filed after the date the issue fee is paid, it must be accompanied by a petition requesting entry and by the fee set forth in Section 1.17(i). If the certified copy is not in the English language, a translation need not be filed except in the case of interference; or when necessary to overcome the date of a reference relied upon by the examiner; or when specifically required by the examiner, in which event an English language translation must be filed together with a statement that the translation of the certified copy is accurate." 37 C.F.R. Section 1.55(a)

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

(complete (d) or (e))

(d) [ ] no such applications have been filed.
(e) [x] such applications have been filed as follows.

NOTE: Where item (c) is entered above and the International Application which designated the U.S. itself claimed priority check item (e), enter the details below and make the priority claim.

PRIOR FOREIGN/PCT APPLICATION(S) FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS APPLICATION AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. SECTION 119(a)-(d)

COUNTRY (OR INDICATE IF PCT)	APPLICATION NUMBER	DATE OF FILING DAY, MONTH, YEAR	PRIORITY CLAIMED UNDER 35 USC 119
IL	130395	9 JUNE 1999	[x]YES [ ]NO
			[ ]YES [ ]NO
			[ ]YES [ ]NO
			[ ]YES [ ]NO
			[ ]YES [ ]NO

CLAIM FOR BENEFIT OF PRIOR U.S. PROVISIONAL APPLICATION(S)
(35 U.S.C. Section 119(e))

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below:

PROVISIONAL APPLICATION NUMBER **FILING DATE**

_____ / _____ _____
_____ / _____ _____
_____ / _____ _____

CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S) UNDER 35 U.S.C. SECTION 120

[ ] The claim for the benefit of any such applications are set forth in the attached ADDED PAGES TO COMBINED DECLARATION AND POWER OF ATTORNEY FOR DIVISIONAL, CONTINUATION OR CONTINUATION-IN-PART (C-I-P) APPLICATION.

ALL FOREIGN APPLICATION(S), *IF ANY*, FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

_____

_____

_____

NOTE: If the application filed more than 12 months from the filing date of this application is a PCT filing forming the basis for this application entering the United States as (1) the national stage, or (2) a continuation, divisional, or continuation-in-part, then also complete ADDED PAGES TO COMBINED DECLARATION AND POWER OF ATTORNEY FOR DIVISIONAL, CONTINUATION OR C-I-P APPLICATION for benefit of the prior U.S. or PCT application(s) under 35 U.S.C. Section 120.

POWER OF ATTORNEY

I hereby appoint the following practitioner(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

(list name and registration number)

JOSEPH H. HANDELMAN, 26179

JOHN RICHARDS, 31053

RICHARD J. STREIT, 25765

PETER D. GALLOWAY, 27885

RICHARD P. BERG, 28145

JULIAN H. COHEN, 20302

WILLIAM R. EVANS 25858

JANET I. CORD, 33778

CLIFFORD J. MASS, 30086

CYNTHIA R. MILLER, 34678

(Check the following item, if applicable)

[ ] I hereby appoint the practitioner(s) associated with the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

[ ] Attached, as part of this declaration and power of attorney, is the authorization of the above-named practitioner(s) to accept and follow instructions from my representative(s).

NOTE: "Special care should be taken in continuation or divisional applications to ensure that any change of correspondence address in a prior application is reflected in the continuation or divisional application. For example, where a copy of the oath or declaration from the prior application is submitted for a continuation or divisional application filed under 37 CFR 1.53(b) and the copy of the oath or declaration from the prior application designates an old correspondence address, the Office may not recognize, in the continuation or divisional application, the change of correspondence address made during the prosecution of the prior application. Applicant is required to identify the change of correspondence address in the continuation or divisional application to ensure that communications from the Office are mailed to the current correspondence address. 37 CFR 1.63(d)(4)." Section 601.03, M.P.E.P., 7th Ed

SEND CORRESPONDENCE TO

Ladas & Parry
26 West 61st Street
New York, N.Y. 10023

DIRECT TELEPHONE CALLS TO:
(Name and telephone number)

JULIAN H. COHEN
(212) 708-1887

(complete the following if applicable)

Since this filing is a [ ] continuation [ ] divisional there is attached hereto a Change of Correspondence Address so that there will be no question as to where the PTO should direct all correspondence.

DECLARATION

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

SIGNATURE(S)

NOTE: Carefully indicate the family (or last) name, as it should appear on the filing receipt and all other document.

NOTE: Each inventor must be identified by full name, including the family name, and at least one given name without abbreviation together with any other given name or initial, and by his/her residence, post office address and country of citizenship. 37 C.F.R. Section 1.63(a)(3).

NOTE: Inventors may execute separate declarations/oaths provided each declaration/oath sets forth all the inventors. Section 1.63(a)(3) requires that a declaration/oath, inter alia, identify each inventor and prohibits the execution of separate declarations/oaths which each sets forth only the name of the executing inventor. 62 Fed. Reg. 53,131, 53,142, October 10, 1997,

Full name of sole or first inventor

1-02

Ilan *(Given Name)* | *(Middle Initial or Name)* | LEVIN *Family (Or Last Name)*

Inventor's signature (x) I. L.K

Date (x) 25.2.2002 **Country of Citizenship** ISRAEL

Residence BEIT DAGAN, ISRAEL ILX

Post Office Address STATE OF ISRAEL-MINISTRY OF AGRICULTURE, VOLCANI RESEARCH CENTER, P.O. BOX 6, 50250 BEIT DAGAN, ISRAEL

Full name of second joint inventor, if any

2-02

Arthur *(Given Name)* | *(Middle Initial or Name)* | SCHAFFER *Family (Or Last Name)*

Inventor's signature (x) Arth D. Schaffer

Date (x) 25.2.02 **Country of Citizenship** ISRAEL

Residence Hashmonaim, Israel ILX

Post Office Address 16 HAZAIT STREET, HASHMONAIM 73127, ISRAEL

Full name of third joint inventor, if any

3-02

Felix *(Given Name)* | *(Middle Initial or Name)* | CINCAREVSKY *Family (Or Last Name)*

Inventor's signature (x) F. Cincarevsky

Date (x) 24.2.2002 **Country of Citizenship** ISRAEL

Residence BEIT DAGAN, ISRAEL ILX

Post Office Address STATE OF ISRAEL-MINISTRY OF AGRICULTURE, VOLCANI RESEARCH CENTER, P.O. BOX 6, 50250 BEIT DAGAN, ISRAEL

(check proper box(es) for any of the following added page(s) that form a part of this declaration)

[ ] **Signature** for fourth and subsequent joint inventors. *Number of pages added* _____

* * *

[ ] **Signature** by administrator(trix), executor(trix) or legal representative for deceased or incapacitated inventor. *Number of pages added* _____

* * *

[ ] **Signature** for inventor who refuses to sign or cannot be reached by person authorized under 37 C.F.R. Section 1.47. *Number of pages added* _____

* * *

[ ] Added page for **signature** by one joint inventor on behalf of deceased inventor(s) where legal representative cannot be appointed in time. (37 C.F.R. Section 1.47)

* * *

[ ] Added pages to combined declaration and power of attorney for divisional, continuation, or continuation-in-part (C-I-P) application.

[ ] Number of pages added _____

* * *

[ ] Authorization of practitioner(s) to accept and follow instructions from representative.

(If no further pages form a part of this Declaration, then end this Declaration with this page and check the following item)

[X] This declaration ends with this page.